

IN VITRO DEMONSTRATION OF CELLULAR SENSITIVITY IN ALLERGIC ENCEPHALOMYELITIS*

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Allergic encephalomyelitis (AE) is a well known experimental disease which can readily be produced in guinea pigs and other animals by the injection of brain and spinal cord in complete Freund's adjuvant (1-6). There are considerable data available to suggest that mechanisms of delayed hypersensitivity may be involved in the pathogenesis of this disease. These include the following: the finding that complete adjuvant is usually needed to induce the disease (7), the early appearance of mononuclear cells in lesions (8), the presence of delayed-type skin reactions in some species (9), and more recently, the transfer of AE from affected to normal animals with lymphoid cells (10-13). A note of caution has been voiced concerning the interpretation of cell transfer studies; it has been pointed out that transfer of the disease could be mediated either by the transferred cells, or by circulating antibody subsequently synthesized by the transferred cells (10, 14). The observation that sera from rabbits with AE produce toxic changes in brain cultures has been taken as evidence to suggest that circulating antibody is the mediator of tissue damage in AE (15). Nevertheless, the inability of sera containing antibody to effect transfer of the disease and, more recently, the demonstration of positive transfer by the intracerebral inoculation of sensitive cells, but not by sera containing antibody (16), provide further support for the view that the sensitive cells themselves may be directly involved in the process of tissue destruction.

In light of the continuing controversy concerning the relative significance of cellular sensitivity and antibody in the pathogenesis of AE, a technique specific for the detection of delayed hypersensitivity, *i.e.* the inhibition of cell migration by antigen, has been applied to the study of this disease. The present paper

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will present data which demonstrate that peritoneal exudate cells taken from guinea pigs with allergic encephalomyelitis exhibit cellular sensitivity *in vitro* to nervous tissue extracts. Further experiments demonstrate that this sensitivity appears to be directed specifically to nervous tissue containing the encephalitogenic antigen(s).

Materials and Methods

Preparation of Tissue Adjuvant Inocula and Injection of Guinea Pigs.—Tissue adjuvant inocula were prepared as previously described (10). One part of guinea pig spinal cord and brain stem was emulsified in two parts phenol water 0.25 per cent. The resulting solution was emulsified with an equal volume of complete Freund's adjuvant containing *Mycobacterium tuberculosis* (strain H37Rv). Guinea pigs were injected with 0.1 ml in each of three foot-pads and 0.1 ml in the ventral aspect of the neck. Emulsions with neonatal rat brain and spinal cord were made up of two parts nervous tissue to one part phenol water in order to increase the final concentration of nervous tissue per inoculation. This solution was then emulsified in an equal volume of complete Freund's adjuvant and injected as above.

Antigen Preparation.—

Guinea pig brain and spinal cord: Two parts of guinea pig brain and one part spinal cord were homogenized in a TenBroeck glass homogenizer with 12 parts minimal essential Eagle's media (MEM, Microbiological Associates, Bethesda, Maryland). This resulted in a 20 per cent suspension of nervous tissue by wet weight. The homogenate was centrifuged at 2000 RPM for 15 minutes. The resultant supernatant was then diluted in MEM. The final media contained 15 per cent guinea pig serum.

The concentration of supernatant used in most experiments was 10 per cent; concentrations greater than 20 per cent proved too cloudy for adequate visualization of cell migration. Control cell migration was unaffected in concentrations of 20 per cent or less. In several experiments dilutions ranging from 10 to 0.1 per cent were used.

Adult rat brain and spinal cord: Prepared as above.

Neonatal rat brain: Tissue was removed within the first 24 hours after birth. A 20 per cent homogenate was prepared as above and centrifuged at 2000 RPM for 15 minutes. It was noted that this tissue contained more water than adult tissue; i.e., after centrifugation in calibrated centrifuge tubes there was less sediment and more supernatant. By comparing the ratios of supernatant and sediment in centrifuged neonatal and adult tissue homogenates, it was possible to determine the amount of neonatal supernatant to use to equalize final concentrations of adult and neonatal tissues in the culture media. Media was also used which contained three times the amount of neonatal nervous tissue as adult nervous tissue. Since results with this concentration were the same as those with equalized concentrations, only the latter have been recorded.

Kidney: Guinea pig and rat kidney were prepared in a similar manner. The kidneys were minced and homogenized 20 per cent by wet weight in MEM in glass homogenizers. The resulting suspension was centrifuged at 2000 RPM for 15 minutes and the supernatant diluted into tissue culture media as above. Solutions containing 5 per cent kidney supernatant were found to be non-toxic to normal cells, and were used throughout.¹

In vitro Test for Delayed Hypersensitivity.—The methods used for placing cells in culture, based on those of George and Vaughan (17), have been described in detail elsewhere (18). Bayol, 30 ml, was injected peritoneally into guinea pigs 9 days after they had been injected with complete Freund's adjuvant and nervous tissue. The peritoneal cells were harvested 72

¹ Solutions containing 10 per cent or more were occasionally toxic to normal cells.

hours later, on the 12th day. The cells were washed twice, made up in tissue culture medium and centrifuged in capillary tubes. The part of the tube containing the packed cells was cut and placed in Mackness-type chambers of 0.8 ml capacity, 2 tubes per chamber. In each experiment chambers were filled with media as follows: two chambers for each dilution of tissue antigen tested and two chambers in media without antigen. The chambers were incubated for 24 hours at 37°C. During this time the cells migrated out of the tubes and onto the glass. The area of migration was projected, drawn, and measured with a planimeter. All data were calculated using the following formula:

$$\frac{\text{Area of migration with antigen}}{\text{Area of migration with no antigen}} \times 100 = \text{per cent migration with antigen}$$

Histological Studies.—Brains and spinal cords were fixed in 10 per cent formaldehyde. Blocks were prepared as previously described (10) and stained in hematoxylin and eosin. Luxol fast blue technique was used for myelin studies.

RESULTS

Effect of Nervous Tissue Extracts on Migration of Cells from Animals with AE.—The migration of peritoneal exudate cells from animals immunized with guinea pig brain and spinal cord was inhibited by extracts of guinea pig nervous tissue when examined after 24 hours of incubation. The results of these experiments are presented in Table I. The average migration of cells from 16 animals with allergic encephalomyelitis was 53 per cent compared to cells from normal animals which migrated an average of 111 per cent ($P = < .001$, see Table II). A second group of control cells were taken from animals injected with complete Freund's adjuvant but without nervous tissue; these migrated 113 per cent in media containing nervous tissue extract. The cells were collected 12 days after immunization at which time many animals exhibited clinical signs of AE (hind limb paralysis and fecal incontinence) while others had not yet developed signs of the disease. There was no apparent correlation between the degree of inhibition of cell migration and overt paralysis of the donor animals at the time cells were harvested. It should be noted however, that the guinea pigs that were not sacrificed all succumbed to AE within 2 to 3 weeks.

Cells from animals with AE which were inhibited by nervous tissue antigen were not inhibited by guinea pig kidney extracts (see Table I and Fig. 1). If anything, the kidney extracts stimulated migration of both normal and sensitive cells. Thus it would appear that the reaction is specific for nervous tissue. High concentrations (20 per cent) of nervous tissue extracts were found to have no inhibitory effect on the migration of normal cells, and at times appeared to stimulate migration in control chambers. This may be a result of the extracts supplementing the nutrients of the media which are probably suboptimal. Twenty per cent nervous tissue supernatant/ml was the highest concentration which still allowed visualization of cell migration. Ten per cent supernatant/ml, which was used in most experiments, markedly inhibited the migration of sensitive cells. It is of note that this amount is probably greatly in excess of the

minimal tissue antigen needed. In 3 experiments concentrations of 2.5 to 0.1 per cent supernatant/ml were as effective as 10 per cent.

Specificity for Encephalitogenic Antigen(s).—At this juncture it was of interest to determine whether the presence of cellular sensitivity in guinea pigs with AE could be more directly correlated with the disease. Reports that neonatal brain in some species does not produce clinical or histological evidence of AE

TABLE I
Effect of Guinea Pig Brain and Kidney Tissue Extracts on the Migration of Peritoneal Cells from Guinea Pigs with Allergic Encephalomyelitis

Migration in antigen*	
Brain‡	Kidney§
<i>per cent</i>	<i>per cent</i>
7	127
26	132
31	138
38	177
40	181
43	
43	
48	
53	
65	
68	
70	
71	
73	
74	
104	
Average 53	151

* In most instances each figure given represents per cent migration of cells from a single animal. In a few cases it was a pool of cells from 2 animals.

‡ Extract present in tissue culture media.

§ Cells migrating in kidney extract come from the same pool of cells which were simultaneously tested and inhibited by guinea pig brain extract.

and probably lacks the encephalitogenic antigen (19, 20) were confirmed using neonatal rat brain. Neonatal rat brain in complete Freund's adjuvant was injected into 24 guinea pigs and 12 rats of the Fisher or Wistar strains. None of the animals developed clinical AE and, in the brains examined, no histological lesions were found. This apparent absence of encephalitogenic antigen in neonatal rat brain made it possible to determine whether cellular sensitivity in AE is directed specifically to the encephalitogenic antigen and not to some other component of brain unrelated to the disease.

TABLE II
The Effect of Guinea Pig Brain and Kidney Extracts on the Migration of Control Cells

Migration in antigen		
Brain*		Kidney*
Normal cells†	Adjuvant cells‡	Adjuvant cells§
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
75	94	104
92	99	117
101	101	130
115	108	160
117	112	
121	113	
122	119	
141	123	
	123	
	134	
Average 111	113	127

* Extracts present in tissue culture media.

† Cells from normal animals.

‡ Cells from animals injected with complete Freund's adjuvant.

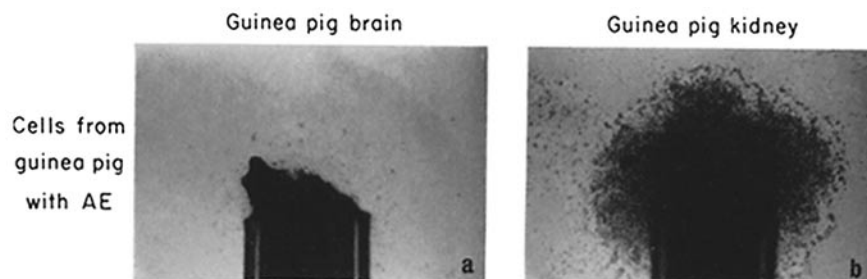


FIG. 1. The effect of extracts of guinea pig nervous tissue on the migration of cells obtained from guinea pigs with allergic encephalomyelitis.

Cells from a guinea pig with allergic encephalomyelitis; (a) inhibited by guinea pig nervous tissue extract, and (b) migrating well in guinea pig kidney extract.

For this series of experiments, cells were obtained from guinea pigs which had been injected with guinea pig nervous tissue. The cells were observed in media containing either guinea pig nervous tissue, adult rat nervous tissue, or neonatal rat nervous tissue. Control chambers without antigen were also prepared. The results are shown on Fig. 2 and Table III. It can be seen that the cells were well inhibited by either guinea pig or adult rat nervous tissue, but

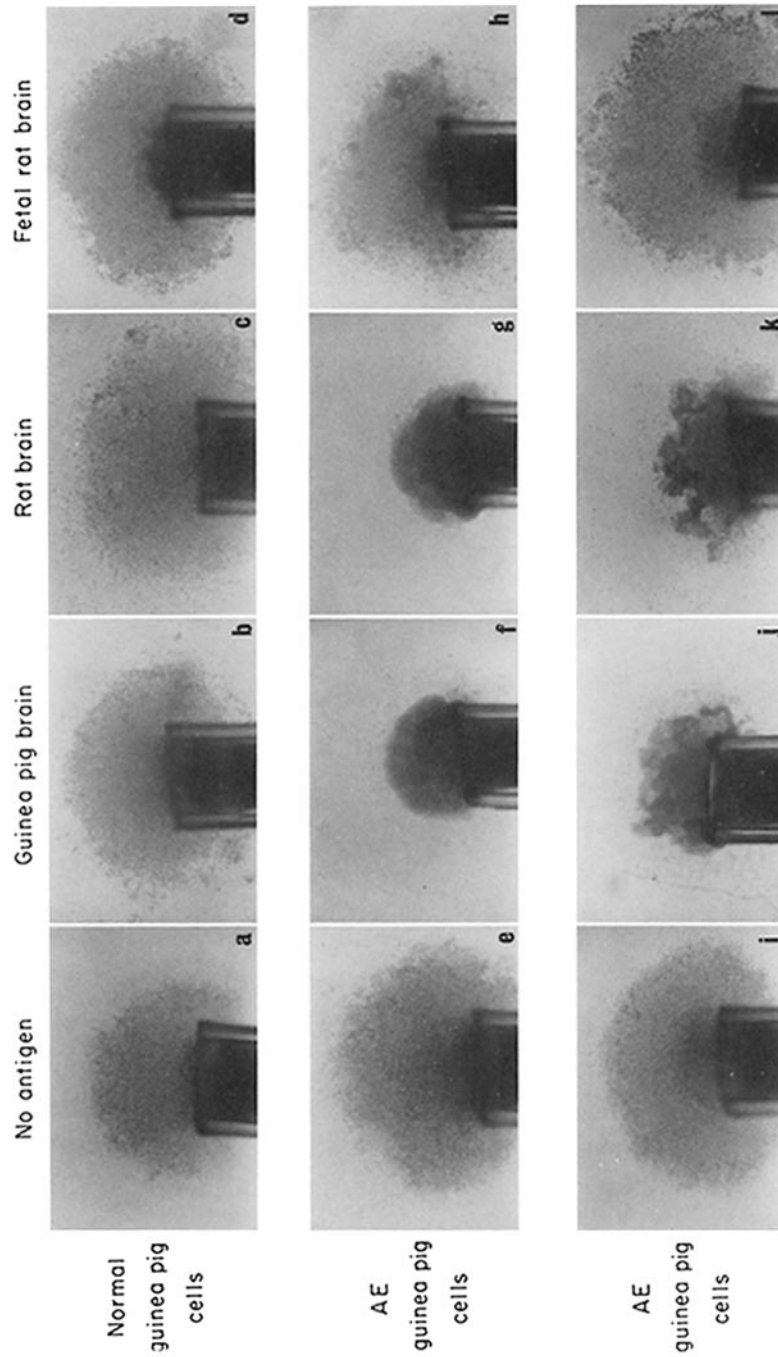


FIG. 2. Effect of nervous tissue extracts on the migration of cells from guinea pigs with allergic encephalomyelitis and control animals. (a) Normal cells in control media; (b) normal cells in guinea pig brain; (c) normal cells in adult rat brain; (d) normal cells in neonatal rat brain; (e) cells from 1 animal with AE in control media; (f) cells from the same animal with AE in guinea pig brain; (g) cells from the same animal with AE in adult rat brain; (h) cells from the same animal with AE in neonatal rat brain; and (i) through (l) cells from another animal with AE in media the same as (e) through (h).

Photographs taken after 24 hours of incubation. X 35.

no inhibition was observed with neonatal rat nervous tissue. This was found in all cases with the exception of one experiment in which cells migrated normally in all three antigens. Previous experiments had shown that adult rat nervous tissue had no effect on normal guinea pig cells. These findings indicate that the cellular sensitivity is directed against a component that appears in maturing nervous tissue parallel with the appearance of encephalitogenic antigen, if not the encephalitogenic antigen itself.

TABLE III
*Effect of Guinea Pig, Adult Rat, and Neonatal Rat Nervous Tissue Extracts on the Migration of Cells from Animals with Allergic Encephalomyelitis**

Guinea pig group	Migration in antigen‡		
	Guinea pig brain§	Adult rat brain§	Neonatal rat brain§
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
AE	48	51	ND
	43	49	114
	43	47	141
	38	42	94
	40	44	145
	104	122	146
Normal	122	119	
	115	132	117

* Induced with guinea pig nervous tissue.

‡ Each horizontal line represents a single experiment in which cells from 1 animal were allowed to migrate in all three antigens.

§ Extract present in tissue culture media.

Experiments were then carried out to determine whether cells from guinea pigs injected with neonatal rat brain would be inhibited by nervous tissue containing encephalitogenic antigen; *i.e.*, guinea pig nervous tissue. The results of these experiments are shown in Table IV. In all cases, the cells migrated well in guinea pig nervous tissue but were inhibited by *both* neonatal rat brain and kidney. The latter was as anticipated since the guinea pigs had been injected with heterologous tissue and might well be expected to develop delayed hypersensitivity to rat tissue components. These findings indicate that cells from guinea pigs injected with neonatal rat nervous tissue do not exhibit sensitivity to the encephalitogenic antigen present in guinea pig nervous tissue. Secondly, they also support the earlier experiments on specificity for they demonstrate that antigens, *i.e.* neonatal rat brain, which did not inhibit the migration of cells obtained from animals with AE clearly do possess antigenic activity in their own right and are capable of inhibiting cell migration.

Attempts to Sensitize Cells to Brain Extracts in Vitro.—Previous attempts to sensitize peritoneal cells with sera from animals exhibiting delayed hypersensitivity to protein antigens or tuberculin have been uniformly unsuccessful (18). It was of interest to see whether this was also the case in AE. Sera from animals whose cells had been markedly inhibited from migrating by nervous tissue extracts were used, after storage at -20°C for 4 weeks. Adjuvant cells (cells from animals injected with complete Freund's adjuvant but not with nervous tissue extracts) were incubated for 30 minutes at 37°C in undiluted sera. The cell concentration was 10 per cent by volume. The cells were washed once and then suspended in tissue culture media containing normal guinea pig sera. Such

TABLE IV
Effect of Guinea Pig or Neonatal Rat Nervous Tissue Extracts on the Migration of Cells from Guinea Pigs Immunized with Neonatal Rat Nervous Tissue

Guinea pig group	Migration in antigen*		
	Guinea pig brain‡	Neonatal rat brain‡	Rat kidney‡
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Injected with neonatal rat brain	119	21	41
	123	22	45
	102	34	64
	123	32	
	112	30	
	113	38	
Normal	117	129	118
	121	92	

* Each horizontal line represents a single experiment in which cells from one animal were allowed to migrate in all three antigens.

‡ Extracts present in tissue culture media.

cells were not inhibited from migrating and in 3 experiments migrated 108, 125, and 152 per cent in nervous tissue extracts. As a necessary control, cells from animals with AE were incubated in normal sera or in the same sera used to incubate adjuvant cells, and then assayed in culture. These cells were still inhibited from migrating by nervous tissue extracts.

DISCUSSION

The present experiments were carried out to determine whether cells obtained from animals with allergic encephalomyelitis exhibited cellular sensitivity to nervous tissue antigens and, more specifically, the encephalitogenic antigen. The interpretation of the results presented depends on the assumption that the *in vitro* test employed is specific for the detection of delayed hypersensitivity. This has been clearly demonstrated in previous studies (17, 18, 22)

which showed that the migration of cells obtained from guinea pigs exhibiting delayed hypersensitivity is inhibited by the specific sensitizing antigen whether or not the animals were also producing circulating antibody. In contrast, cells obtained from animals which are synthesizing antibody but not exhibiting delayed hypersensitivity are not inhibited by antigen. In addition, numerous attempts to sensitize cells with sera obtained from animals with delayed hypersensitivity or sera containing antibody have been unsuccessful.

In light of these observations, the finding that the migration of peritoneal cells obtained from animals with AE is inhibited specifically by extracts of brain and spinal cord clearly indicates that these animals display cellular sensitivity to some nervous tissue antigens.

The detection of sensitive cells in animals with AE, however, does not of itself prove a pathogenic role for these cells anymore than the previous detection of complement-fixing (CF) antibodies to brain indicated that these mediate the tissue destruction in AE. On the contrary, no correlation was found between the appearance of CF antibodies and the occurrence of AE (4). Furthermore, the CF antibodies were directed to a non-paralytic antigen(s) and appeared to play no direct role in the induction of the disease (23). Thus it was important to determine whether the cellular sensitivity observed in animals with AE was directed specifically to the encephalitogenic antigen(s), and not to other components of brain unrelated to the disease before a pathogenic role for sensitive cells could be implied.

Rat nervous tissue appeared to be an ideal antigen with which to study this problem of specificity. Adult and neonatal rat nervous tissue must have numerous antigens in common yet they differ significantly in at least one. Adult rat nervous tissue can produce AE and thus contains the encephalitogenic antigen, while neonatal rat nervous tissue does not produce AE, and lacks detectable amounts of the encephalitogenic antigen. This antigen is thought to be a myelin constituent; the paralytic properties of nervous tissue have been shown to correlate with the laying down of myelin (19, 20). More recently, the antigen has been localized in myelin by immunofluorescent techniques (21). The experiments reported here utilizing rat nervous tissue strongly suggest that cellular sensitivity in animals with AE is indeed specific for the encephalitogenic antigen. The migration of cells obtained from guinea pigs with AE was inhibited by nervous tissue which is capable of producing the disease (guinea pig or adult rat brain and spinal cord) but was not inhibited by neonatal rat nervous tissue lacking encephalitogenic properties. Furthermore, guinea pigs injected with neonatal rat nervous tissue in complete adjuvant did not develop AE, and the migration of cells from these animals was not inhibited by nervous tissue containing the encephalitogenic antigen; *i.e.*, guinea pig brain and spinal cord.

It would appear that one or more components come into being in the neonatal rat brain as it matures which are capable both of producing AE and of inhibiting

the migration of peritoneal cells obtained from animals with AE. In all likelihood, these components are the same. However, an alternative possibility, both complicated and unlikely, cannot be ruled out, that several materials develop in the maturing brain only some of which are encephalitogenic and that cellular sensitivity is directed against one of these newly formed substances which is not the encephalitogenic antigen.

The finding that cells obtained from guinea pigs with AE display cellular sensitivity to brain antigen and that this sensitivity seems to be directed specifically to the encephalitogenic antigen(s) serves to underline the possibility that cells with such a highly specific sensitivity could well play an important role in the pathogenesis of allergic encephalomyelitis.

SUMMARY

Peritoneal cells obtained from animals exhibiting delayed hypersensitivity are inhibited from migrating *in vitro* by specific sensitizing antigen. This test for the detection of delayed hypersensitivity was applied to the problem of cellular sensitivity in allergic encephalomyelitis (AE). The migration of peritoneal cells obtained from guinea pigs with AE was inhibited specifically by nervous tissue antigens. The specificity of this reaction was further studied. Neonatal rat nervous tissue, which was shown to lack the encephalitogenic antigen, *i.e.* did not produce AE when injected with complete adjuvant into guinea pigs and rats, did not inhibit the migration of cells from animals with AE. Adult rat nervous tissue, which readily produces AE, and thus contains the encephalitogenic antigen did inhibit the migration of such cells. The finding that cells from animals with AE display hypersensitivity which appears to be directed specifically to the encephalitogenic antigen strongly supports the view that such cells could play an important role in the pathogenesis of this disease.

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BIBLIOGRAPHY

1. Morgan, I. M., Allergic encephalomyelitis in monkeys in response to injection of normal monkey nervous tissue, *J. Exp. Med.*, 1947, **85**, 131.
2. Kabat, E. A., Wolf, A., and Berger, A. E., The rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants, *J. Exp. Med.*, 1947, **85**, 117.
3. Freund, J., Stern, E. R., and Pisani, T. M., Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion, *J. Immunol.*, 1947, **57**, 179.
4. Paterson, P. Y., Organ-specific tissue damage induced by mammalian tissue-adjuvant emulsions, in *Cellular and Humoral Aspects of the Hypersensitive States*, (H. S. Lawrence, editor), New York, Paul B. Hoeber, 1959, 469.
5. Waksman, B. M., Experimental allergic encephalomyelitis and the "autoal-

- lergic" diseases, *Internat. Arch. Allergy and Appl. Immunol., Suppl.*, 1959, **14**, 1.
6. Kies, M. W., and Alvord, E. C., Jr., "Allergic" Encephalomyelitis, Springfield, Illinois, Charles C. Thomas, 1959 (symposia).
 7. Freund, J., Sensitization with organ specific antigens and the mechanisms of enhancement of the immune responses, *J. Allergy*, 1957, **28**, 18.
 8. Waksman, B. M., and Adams, R. D., A histologic study of the early lesions in experimental allergic encephalomyelitis in the guinea pig and rabbit, *Am. J. Path.*, 1962, **41**, 135.
 9. Waksman, B. M., and Morrison, L. R., Tuberculin type sensitivity to spinal cord antigen in rabbits with isoallergic encephalomyelitis, *J. Immunol.*, 1951, **66**, 421.
 10. Paterson, P. Y., Transfer of allergic encephalomyelitis in rats by means of lymph node cells, *J. Exp. Med.*, 1960, **111**, 119.
 11. Astrom, K. E., and Waksman, B. M., The passive transfer of experimental allergic encephalomyelitis and neuritis with living lymphoid cells, *J. Path. and Bact.*, 1962, **83**, 89.
 12. Stone, S. M., Transfer of allergic encephalomyelitis by lymph node cells in inbred guinea pigs, *Science*, 1961, **134**, 619.
 13. Koprowski, H., The role of hyperergy in measles encephalitis, *Am. J. Dis. Child.*, 1962, **103**, 273.
 14. Chase, M. W., A critique of attempts at passive transfer of sensitivity to nervous tissue, in "Allergic" Encephalomyelitis, (M. W. Kies and E. C. Alvord, Jr., editors), Springfield, Illinois, Charles C. Thomas, 1959, 348.
 15. Bornstein, M. B., and Appel, S. N., The application of tissue culture to the study of experimental "allergic encephalomyelitis." I. Patterns of demyelination, *J. Neuropath. and Exp. Neurol.*, 1961, **20**, 141.
 16. Paterson, P. Y., and Weiss, H. S., Transfer of allergic encephalomyelitis in rats by intracerebral injection of lymphoid cells, *Proc. Soc. Exp. Biol. and Med.*, 1965, **119**, 267.
 17. George, M., and Vaughan, J. M., *In vitro* cell migration as a model for delayed sensitivity, *Proc. Soc. Exp. Biol. and Med.*, 1962, **111**, 514.
 18. David, J. R., Al-Askari, S., Lawrence, H. S., and Thomas, L., Delayed hypersensitivity *in vitro*. I. The specificity of inhibition of cell migration by antigens, *J. Immunol.*, 1964, **93**, 264.
 19. Kabat, E. A., Wolf, A., and Berger, A. E., Studies on acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants, *J. Exp. Med.*, 1948, **88**, 417.
 20. Waksman, B. M., Allergic encephalomyelitis in rats and rabbits pretreated with nervous tissue, *J. of Neuropath. and Exp. Neurol.*, 1959, **18**, 397.
 21. Rauch, H. C., and Raffel, S., Immunofluorescent localization of encephalitogenic protein in myelin, *J. Immunol.*, 1964, **92**, 452.
 22. David, J. R., Lawrence, H. S., and Thomas, L., Delayed hypersensitivity *in vitro*. III. The specificity of hapten-protein conjugates in the inhibition of cell migration, *J. Immunol.*, 1964, **93**, 279.
 23. Paterson, P. Y., A study of experimental encephalomyelitis employing mammalian and nonmammalian nervous tissues, *J. Immunol.*, 1957, **78**, 472.